J Young Pharm, 2018; 10(1): 48-51
A multifaceted peer reviewed journal in the field of Pharmacy
www.jyoungpharm.org | www.phcog.net

Original Article

Analysis of Rifampicin in Dried Blood Spot of Tuberculosis Patients for Therapeutic Drug Monitoring using High Performance Liquid Chromatography

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ABSTRACT

Objective: This research aimed to determine rifampicin in dried blood spot of tuberculosis patients using cilostazol as internal standard by high performance liquid chromatography for supporting therapeutic drug monitoring. **Method:** Analytes were extracted by protein precipitation using methanol-acetonitril (4:1). The analytical separation was performed on C-8 column (Waters, Sun fire™ 5 µm; 250 x 4.6 mm) with column temperature of 40°C. The mobile phase used was acetonitrile - methanol - ammnonium acetate buffer pH 4.5 (40:30:30 % v/v) with a flow rate of 0.5 mL/min; and detected at 261 nm. **Results:** The method had a chromatographic run time of 16 min and linear calibration curve over the range of 1-30 µg/mL with a correlation coefficient (r) of 0.998. The result of the analysis of rifampicin in 18 tuberculosis patients showed that the measured value of rifampicin was in the range of 1.26 to 18.33 µg/ml. **Conclusion:** Based on the result, the concentration of rifampicin in patients were varied. There were 7 of 18 patients which had concentration in therapeutic range, showing that the treatment is appropriate, while 11 of 18 patients had concentration below the therapeutic range showing that dose adjustment is needed.

Key words: Rifampicin, HPLC, Tuberculosis, Validation, Dried blood spot.

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DOI: 10.5530/jyp.2018.10.12

INTRODUCTION

Tuberculosis is an infection disease caused by *Mycobacterium tuberculosis*. The disease generally attacks lung and is transmitted through coughing and sneezing. The appearing symptoms are coughing with blood, chest pain, sweating at night, fever for more than 1 month, weight loss, and anorexia.[1] World Health Organization, estimated that 8.8 million people were infected with TB in 2010.[2] Indonesia is one of the countries with high risk of TB in Southeast Asia. Among 500,000 cases occurs every year, 175,000 of humans had died. *Antituberculosis* first line drugs are rifampicin, pyrazinamide, ethambutol, and isoniazid. Mycobacteria caused by TB can colonize in the cell and develop the ability of resistance to fight *antituberculosis* and certain drugs. To prevent that condition, treatment of TB must consist of 2 more drugs to prevent a resistance and reduce its side effect.[3] Mycobacteria response to drugs is very slow therefore treatment should also lasts more than 2 months until one year. Combination of first line *antituberculosis* is present in the form of fixed dose combination.[4]

Rifampicin is an antibiotic from macrolide group, which blocks the initial conformation of RNA chain of the bacteria and inhibits the growth of positive g and negative g bacteria. Rifampicin is absorbed rapidly in the GIT, distributed to whole body in effective concentration to many organs and body fluids, including cerebrospinal fluids.[5] Rifampicin is an antibiotic type which can cause a drug resistance. The resistance organism can hold drug activity, so the standard of treatment is not effective, and the infection will be persistent and spread. Therapeutic range of rifampicin is 8-24 µg/ml and a dosage increace is recommended if the concentration of rifampicin is less than 6 µg/ml.[5]

Therapeutic drug monitoring (TDM) is a part of clinical practice for measuring specific drugs at designated intervals to maintain a constant concentration in a patient's bloodstream, thereby optimizing individual dosage regiments. To know the effectiveness of a treatment, there should be determination of drug concentration around maximum concentration and elimination phase.[6,7,8] Therapeutic effect of rifampicin was determined with drugs concentration in blood.

Biosampling method with dried blood spot and absorb paper had some advantages such as less invasive, only small volume needed, a relatively stable analyte, easy in distribution and storage, does not need a phlebotomist, reduce the risk of infection and cost-effective.[9] To provide a reliable therapeutic drug monitrong method, rifampicin was analyzed in dried blood spot tuberculosis patients, who consume rifampicin and isoniazid in fixed dose combination at Bekasi general hospital. This research had accepted an ethical clearance from ethical committee at medical faculty Universitas Indonesia No 1102 UN2/F1/ETIK/2016.

MATERIALS AND METHODS

Chemical and Reagents

Rifampicin (U.S. Pharmacopeia, Rockville, U.S.), isoniazid (Zhejiang Jiangbei Pharmaceutical Co. Ltd, Taizhou, China), cilostazole as an internal standard (Assia Chemical Industries Ltd, Beer-Sheva, Israel), HPLC grade acetonitrile, HPLC grade methanol, ammonium acetate, and sodium dihydrogen phosphate (Merck), aquabidest and ascorbic acid from Brataco, Perkin Elmer 226 as the DBS card (Perkin Elmer, Massachusetts, U.S.), and whole blood from Indonesian Red Cross.

Instruments

High Performance Liquid Chromatography (Shimadzu, LC-20AD) equipped with pump, degasser (Shimadzu, DGU-20A_5), C-18 (Waters,

Sunfire™ 5 µm; 250 x 4.6 mm), C-8 (Waters, Sunfire™ 5 µm; 250 x 4.6 mm), UV-Vis detector (Shimadzu, SPD-20A), column oven (Shimadzu, CTO-10AS vp), autosampler (Shimadzu, SIL-20A), and data processor (Lab Solutions), spectrophotometer UV (Jasco), evaporator (Turbo Vap LV), ultrasonicator (Elmasonic), pH meter (EUTECH), vortex (Maxi Mix II), centrifugator (Digisystem), microcentrifugator (Spectrafuge 16M), micropipette Eppendorf (Soccorex).

Chromatographic Condition

This study was conducted using C-8, 5 µm, 250 x 4.6 mm by HPLC. The mobile phase consisted of 50 mM ammonium acetate buffer, pH 4.5 – acetonitrile – methanol (40:30:30) with an isocratic elution. Injection volume was 20 µl. Detection was carried out at 261 nm, the column temperature was 40°C, and the flow rate was 0.5 ml/min.

Preparation of Standard Solution

10.0 mg rifampicin and 5.0 mg ascorbic acid was weighed then dissolved in the 50% methanol in 10 mL volumetric flask to obtain 1.0 mg/mL (1000 ppm) concentration. A 10.0 mg cilostazole was weighed and was dissolved with 50% methanol in 10.0 mL volumetric flask to obtain 1.0 mg/mL (1000 ppm) concentration. The dilution was conducted to obtain solutions in certain concentrations.

Preparation of Mobile Phase

Ammonium acetate for 1.927 g in 500 mL water, then orthophosphoric acid was added to make a pH value of 4.5. The solution was mixed with acetonitrile and methanol to obtain acetonitrile–methanol-ammonium acetate buffer mixtures in 30:30:40 ratio.

Sample Preparations

A 1000 µL whole blood which contain 1 µg/mL rifampicin were mixed with 50 µl cilostazol as internal standard with concentration of 10 µg/mL. The solution was added with 1 mL acetonitrile and methanol (4:1) then vortex mixed for 2 minutes. The tube was then centrifuged for 15 min at 10.000 rpm. Organic phase was transferred to another tube and evaporated with Nitrogen gas for 20 minutes at 35°C. The residue was then reconstituted with 200 µL mobile phase. The aliquot was injected into the HPLC system.

Method Validation of Rifampicin in Dried Blood Spot

In this study the method validation refers to EMEA guideline for bioanalytical method validation.[10] Full validation of rifampicin analytical method in dried blood spot was conducted in term of parameters LLOQ, selectivity, accuracy, precision, and recovery, carry over, stability parameters, linear calibration curve, and dilution integrity.

RESULTS

Method Validation of Rifampicin in Dried Blood Spot Selectivity

Selectivity test was conducted in the blank DBS and LLOQ concentrations were obtained using plasma from six different sources. The study results showed no interferences peak in the retention time of the analyte and the internal standard.

Carry Over

The study showed no carry over effect in the blank DBS after injecting the highest concentration (ULOQ) of rifampicin. The carry over percentage still meets the requirements for analyte (<20%) and IS (<5%).

Calibration Curve and LLOQ

Calibration curve was linear with the correlation coefficient (r>0.9980) in concentration range from 1.0 to 30 µg/mL. LLOQ concentration of rifampicin was 1.0 µg/mL with the CV value of 6.22 % and % diff range between -0.92 to 15.68%.

Accuracy, Precision, and Recovery

Accuracy and precision were conducted in within run and between run on 4 different concentrations, such as LLOQ, QCL, QCM, and QCH. The accuracy and precision results of the within-run and between-run are shown in Table 2. The recovery was conducted to compare the analyte peak respond in the DBS sample with the standard solution. The results of within-run and between-run precision and accuracy of rifampicin were less than 15% for CV and 20% for bias. The bias in within-run and between-run accuracy was 14.38% to 1.10% and 7.76% to 11.24%.

Dilution Integrity

Dilution integrity was conducted to determine the accuracy, precision, and reliability of the dilution in bioanalytical process. If the *in vivo* rifampicin assay in the biologic matrix obtained more than the highest requirement in 30 µg/mL, a dilution process should be conducted to provide a desirable calibration curve range.

Stability

The storage stability of rifampicin in DBS paper was evaluated to determine whether degradation occurred during long-term storage. Stability was determined by analyzing QC sample stored at room temperature over a period of 40 days. The data indicated that rifampicin was stable at room temperature for at least 40 days with bias of 12.54% and 14.76% on two concentration levels (QCL and QCH) respectively.

Sampling Process on Tuberculosis Patients

This study was approved (no: 1102 UN2/F1/ETIK/2016) by the Ethics Committee of Faculty of Medicine, Universities Indonesia. Prior to the study, the patients had signed the informed consent. The samples were dried blood spot of 18 tuberculosis patients who received rifampicin containing isoniazid in fixed dose combination in their anti-tuberculosis regiment. The received dosage was 300-600 mg/m^2 of rifampicin. The patients fulfill the inclusion criteria such as:

a. patients of Bekasi General Hospital
b. receives rifampicin in fixed dosed combination with isoniazid
c. patient's age is 18-50 years old during the blood collection
d. patient is willing to take part in the research and sign the informed consent

Blood sample from patients as much as 100 µl were collected at 2 h and 6 h after administration of rifampicin, the blood was spotted on the DBS paper and was dried for 3 h. Then DBS paper was inserted to a zip lock bag and stored at room temperature until analysis was conducted.

Analysis of Study Sample

The rifampicin level was found on 18 patients sample with the lowest level of 3.7 µg/ml on patient SN04 and the highest level of 18.33 µg/ml on patient SN14 at 2 h after administration of rifampicin. Blood sampling 6 h after administration of rifampicinin showed the lowest level of rifampicin which was 1.26 µg/ml on patient SN08 and the highest level of rifampicin which was 15.15 µg/ml on patient SN14. The average level of rifampicin at 2 h after administration of rifampicin was 9.01 µg/ml and the average of rifampicin after 6 h after administration of rifampicin was 7.01 µg/ml.

DISCUSSION

The results of this research were in accordance to the previous research which showed that blood sampling 2 h after administration of rifampicin

shown the rifampicin at maximum concentration and blood sampling 6 h after administration of rifampicin shown the rifampicin at elimination phase.[11] Meanwhile, Unsalan *et al* reported a different result showing that maximum concentration of rifampicin was seen at 3h after administration of rifampicin and therapeutic range of rifampicin is 8-24μg/ml.[12]

Table 1: Table of selectivity test.

concentration (μg/mL)				Interferent (%)
	WB	RIF	Blanko	
1.00	A	2901	114	3.93
		3083	138	4.48
	B	2756	129	4,68
		2400	141	5.88
	C	2186	268	12.26
		2575	251	9.75
	D	2821	376	13.33
		3336	368	11.03
	E	294	135	5,20
		2763	129	4.67
	F	2686	289	10.76
		2313	291	12.58

The analysis result of 18 patients tuberculosis showed rifampicin at maximum concentration in 11 samples were in therapeutic range at 2 h after administration. At 6 h after administration of rifampicin, 8 samples had concentration in therapeutic range, while 7 patients had concentration in therapeutic range after 2 h and 6 h after administration of rifampicin. It shows that the treatment is appropriate, and rifampicin is effective. Meanwhile, 6 patients did not meet the therapeutic concentration range at 2 h and 6 h after administration of rifampicin. This results indicate that the treatment had failed because of several factors and one of them is drugs resistance. It can be concluded that the regiment need dose adjustment. Four patients had rifampicin concentration at 2h after administration in therapeutic range, but had rifampicin concentration below therapeutic range at 6 h after administration of rifampicin. It showed that this group of patients had abnormalities in GIT absorption (malabsorption) and drugs interaction. One patient had rifampicin concentration below therapeutic range at 2 h after administration and had rifampicin concentration at the therapeutic range at 6 h after administration. Moreover, 3 patients had lower concentration at 2 h after administration than 6 h after administration. This results indicates a delayed absorption.[7]

Table 3 showed the correlation of rifampicin dose with its concentration in plasma. This concentration difference may be caused by the difference of rifampicin dose. However, the result showed that there was no guaranteed a higher dose of rifampicin will give high concentration in plasma. In general, patients had 450 mg of rifampicin dosage and 38-54 kg weight range.

In previous research conducted by Vu *et al*, in the determination of rifampicin in plasma and dried blood spot has been compared and had

Table 2: Accuracy and precision within-run of rifampicin.

Concentration (μg/mL)	Area (μV.s)		PAR	Measured value				
	Rifampicin	IS		Concentration (μg/mL)	Average (μg/mL)	SD	% CV	% Diff
1.00	2511	197729	0.0127	1.11	1.12	0.05	4.77	10.75
	2629	189975	0.0138	1.19				19.27
	2882	216994	0.0133	1.15				15.10
	2578	200448	0.0129	1.12				11.96
	2806	235730	0.0119	1.05				4.80
3.00	9823	252497	0.0389	3.07	3.02	0.28	9.42	2.16
	8802	258832	0.0340	2.70				-9.78
	8582	247645	0.0347	2.75				-8.20
	8599	208382	0.0413	3.24				7.92
	9357	220590	0.0424	3.33				10.73
12.75	37033	249218	0.1486	11.27	11.63	0.57	4.92	-11.64
	32200	221438	0.1454	11.03				-13.51
	36056	238991	0.1509	11.44				-10.31
	31142	189689	0.1642	12.43				-2.51
	31383	198150	0.1584	12.00				-5.90
22.5	59555	216782	0.2747	20.69	22.77	2.13	9.35	-8.03
	58438	214126	0.2729	20.56				-8.63
	54603	161882	0.3373	25.37				12.76
	53583	175101	0.3060	2303				2.37
	55229	171611	0.3218	24.21				7.62

Table 3: Accuracy and precision between-run.

LLOQ		Within-run					Between- run		
Concentration (µg/mL)	Day	Concentration (µg/mL)	Mean (µg/mL)	SD	% CV	% Diff	Mean (µg/m L)	SD	% CV
	1	1.03				3.44			
		0.97				-3.03			
		0.82	0.96	0.08	8.36	-17.88			
		0.98				-2.38			
		0.98				-1.74			
	2	1.08				8.02			
		0.87				-13.01			
1.00		1.16	1.03	0.11	10.74	15.61	1.01	0.12	11.40
		1.07				6.70			
		0.97				-2.84			
	3	1.19				19.37			
		1.14				13.90			
		0.90	1.04	0.15	14.38	-10.16			
		1.12				11.53			
		0.86				-13.52			

a linear concentration of r=0.95. Hence, there is no significant difference in determining rifampicin in plasma and dried blood spot and it did not need conversion factors to convert rifampicin concentration in dried blood spot to plasma.[13] Therefore, bio sampling method with dried blood spot give more advantages compared to plasma for therapeutic drug monitoring because it is less invasive, only small volume needed, a relatively stable analyte, easy in distribution and storage, no phlebotomist needed, reduce the risk of infection and cost-effective.

CONCLUSION

The analytical method of dried blood spot was linear at concentration range of 1 – 30 µg/mL and the level of rifampicin in 18 of tuberculosis patients was in the range of 1.26 to 18.33 µg/ml. Based on the result, the concentration of rifampicin in patients were varied, there were 7 of 18 patients had concentration in therapeutic range, which shows that the treatment is appropriate. While 11 of 18 patients had concentration below the therapeutic range, which shows that dosage adjustment is needed.

ACKNOWLEDGMENT

1. Bekasi General Hospital which provides the tuberculosis patients
2. Directorate of Research and Community Services Universitas Indonesia who gives grants for this research.

CONFLICT OF INTEREST

No conflict of interest is declared.

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Article History: Submission Date : 11-08-2017 ; Revised Date : 30-09-2017; Acceptance Date : 04-11-2017.
Cite this article: Harahap Y, Alkindy F, Ashiila G, Rahmayanti. Analysis of Rifampicin in Dried Blood Spot of Tuberculosis Patients for Therapeutic Drug Monitoring using High Performance Liquid Chromatography. J Young Pharm. 2018;10(1):48-51.